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Pulmonary Function Changes in Experimental Graft-versus-Host Disease of the Lung

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ABSTRACT

Pulmonary graft-versus-host disease (p-GVHD) is a serious complication after allogeneic stem cell transplantation (allo-SCT) of high morbidity and high mortality. We characterized breathing patterns and pulmonary function changes in correlation to lung histopathology and survival by using a well-established murine model of p-GVHD. Lethally irradiated B6D2F1 mice received SCT from either syngeneic B6D2F1 or allogeneic C57BL/6 animals. Within 6 weeks, severe p-GVHD developed in allogeneic recipients characterized by progressive interstitial, alveolar, peribronchial, and periluminal inflammatory cell infiltration, whereas in syngeneic recipients lung histology remained normal. Allogeneic recipients demonstrated decreased minute ventilation (MV), reduced peak inspiratory and expiratory flow rates as early as 1 week after SCT. In addition, allo-SCT resulted in restrictive pulmonary function changes as early as 7 days after transplantation and in progressive airflow obstruction within 6 weeks. Decreased breathing abilities and pulmonary function changes of allogeneic recipients were associated with increased mortality and the severity of acute graft-versus-host disease (aGVHD). These findings show that p-GVHD can be characterized by changes in pulmonary function and functional respiratory insufficiency. Furthermore, our data strengthen the understanding, that the lung is a critical target organ of aGVHD.

KEY WORDS

Allogeneic stem cell transplantation • Lung • Graft-versus-host disease • Pulmonary function • Restrictive • Obstructive

INTRODUCTION

Allogeneic (allo-) stem cell transplantation (SCT) is an important therapeutic option for a number of malignant and nonmalignant diseases. However, the utility of allo-SCT is limited by severe complications, including the development of acute graft-versus-host disease (aGVHD) and pulmonary injury. Historically, approximately 50% of all pulmonary infiltrates seen after SCT have been secondary to infection, but the judicious use of broad-spectrum antimicrobial prophylaxis in recent years has tipped the balance of pulmonary complications from infectious to noninfectious causes [1]. In addition to periengraftment syndrome (PERDS), a noninfectious, alloantigen-independent injury occurring around the time of engraftment [2], various forms of noninfectious, alloimmune-mediated acute lung injuries can develop within the first few months after allo-SCT, such as interstitial and alveolar pneumonitis, perivascular lymphocytic inflammation, and lymphocytic bronchiolitis, and are rarely seen in patients after autologous SCT [3-8]. The most dramatic clinical course has been associated with noninfectious interstitial pneumonitis classically defined as idiopathic pneumonia syndrome (IPS) [4]. IPS occurs in 5% to 25% of patients after allo-SCT, depending upon conditioning toxicity, specifically the use of lethal total body irradiation (TBI), donor source, and the degree of antigenic mismatch between donor and recipient, and has a mortality of approximately 80% [9,10]. Animal models showed that the underlying pathophysiology is complex, and involves conditioning-related cytotoxic effects [9],

inflammatory cytokine production [11-14], and the recruitment of both donor cytotoxic and helper T cells and accessory cells into the lung [13-20]. Therefore, concurrent understanding grows, that IPS and other overlapping forms of acute noninfectious lung injury after allo-SCT resemble forms of acute pulmonary GVHD (p-GVHD) and p-GVHD-related toxicity.

Various models of acute lung injury mirroring acute p-GVHD after allo-SCT have been described [14,15,17,20,21]. We extensively studied the underlying mechanisms of donor leukocyte recruitment into the lung using a murine model of allo-SCT, in which donor cell alloreactivity developed across MHC class I, MHC class II, and minor antigen mismatches, and in which p-GVHD was reliably induced within 3 to 6 weeks after transplantation [13,17-19]. In these studies as well as others, alterations in histopathology, pulmonary vascular endothelial cell integrity, and the cellular and cytokine content of bronchoalveolar lavage fluid correlate very well with that seen in patients [3,4,13-16,22,23], and have been the hallmark of experimental lung injury, but pulmonary function tests (PFT) have been only sporadically used to determine the extent of disease [14,16,24-26]. Especially, no serial analysis of breathing characteristics has yet been performed to assess the kinetics of pulmonary impairment incurred during the clinical course post-allo-SCT.

In the present study, we determined the kinetics of SCT-related changes of breathing patterns, including respiratory rate (RR), tidal volume (TV), minute ventilation (MV), peak inspiratory flow (PIF), and peak expiratory flow (PEF), as well as alterations of lung volumes and forced expiration maneuvers in our previously characterized allo-SCT model [17]. Changes in pulmonary function were correlated to lung histopathology and to survival. As expected, allo-SCT resulted in severe p-GVHD development, which was clinically paralleled by respiratory insufficiency and increased mortality.

MATERIALS AND METHODS

Mice and Stem Cell Transplantation

Female C57BL/6 (H-2^{b}) and B6D2F1 (H-2^{bxd}) mice were purchased from Charles River Laboratories (Sulzbach, Germany) and acclimatized in our animal facility for at least 1 week before the experiments. Animals were between 10 and 20 weeks old at the time of SCT. All animal experiments were approved by the local institutional animal committee of the University of Regensburg, and were in accordance with German animal protection laws.

Mice were transplanted according to a standard protocol as previously described [17]. On the day of SCT, B6D2F1 recipient mice received lethal TBI delivered in 2 fractions 3 hours apart to reduce gastrointestinal toxicity. TBI was given by a linear accelerator at a dose rate of 150 cGy/min. A total dose of 13 Gy was given to animals used to assess lung volumes, forced ventilation parameters, and histopathology, and 12 Gy were given to those animals used for weekly measurements of breathing patterns within in the same animal up to 12 weeks after allo-SCT. TBI was followed by the infusion of cell mixtures of 5×10^6 bone marrow cells supplemented with 1, 3, or 6×10^6 splenocytes from either syngeneic (B6D2F1) or allogeneic (C57BL/6) donors. Transplanted mice were housed in microisolator cages and received autoclaved chow, bedding, and hyperchlorinated water ad libitum.

Clinical GVHD and Survival

Survival was monitored daily until day +84 after SCT, and clinical GVHD scores were assessed weekly by a scoring system incorporating 5 clinical parameters: weight loss, posture (hunching), mobility, fur texture, and skin integrity, as described previously [15]. Each parameter was graded between 0 and 2. Once an animal reached a cumulative score $\geq$6.5 or a weight loss of >30% of the initial weight before SCT, the mouse was sacrificed and counted as a GVHD-related death.

Measurement of Lung Function

Assessment of pulmonary function was performed using a Buxco lung function analysis system (BUXCO Electronics, Troy, NY) consisting of a pulmonary function test/forced maneuvers analyzer (SFT3840) plus pressure panel for mouse maneuvers (AUT6100), an unrestrained whole-body plethysmograph (WBP) (SFT3812), and an anesthetized mouse—PFT plethysmograph (PLY3112). Data acquisition and analysis was done using BioSystem XA software (SFT3850).

The use of the unrestrained WBP allows the repeated measurement of respiratory patterns in conscious, freely moving animals at multiple time points. Measurements were performed according to the manufacturer's on-site instruction and protocol. Each animal was tested individually. Prior to testing, the mouse was allowed to acclimatize to the chamber for at least 2 minutes until no signs of distress were evident and the animal appeared relaxed and calmly breathing. Data acquisition was performed over a time period of 2 to 3 minutes, during which the spontaneously breathing animal moved freely in the chamber. In vivo parameters of breathing obtained included: TV (mL), RR (breaths per minute), MV (mL/min), PIF (mL/sec) and PEF (mL/sec). For each individual mouse this test was repeated 3 times in sequence and values were averaged. For kinetics, animals were analyzed before SCT and then weekly thereafter until day +84. Data after SCT were presented in relation to pre-SCT values of the same animal by using the formula: % of pre-SCT value = measured value after SCT/pre-SCT value $\times$ 100.

The anesthetized mouse - PFT plethysmograph, allows the assessment of lung volumes and forced

ventilation parameters. Measurements were performed according to the manufacturer's on-site instruction and protocol. To perform these tests, animals were anesthetized by the intraperitoneal injection of ketamine hydrochloride (50 mg/kg) and medetomidine hydrochloride (0.33 mg/kg). The trachea was dissected, cleaned of surrounding tissue, and cannulated with a 17- to 19-gauge cannula, depending on the trachea size and the size of the mouse. Animals were placed into the chamber and mouse lungs were connected over the tracheal cannula through a port in the chamber wall to a built-in ventilator. Sedation was ensured to be deep enough to prevent spontaneous breathing against passive ventilation before tests were performed. Fast flow volume measurements were done to assess air outflow obstruction, and quasistatic pressure volume measurements were performed to assess lung volumes. Parameters included in the analysis were vital capacity (VC, μL), forced vital capacity (FVC, μL), forced expiratory volume (FEV, μL) at 20, 50, 100, and 200 milliseconds, forced expiratory flow (FEF, mL/sec) at 25% of expiration, and chord complicance at 0-10 cm H_2O (Cchord, mL/cm H_2O). As animals were sacrificed following these procedures, fast flow volume measurements and quasistatic pressure volume measurements could not be repeatedly performed for kinetics within the same animal, but required different animals for data acquisition at different time points after SCT. Measurements were conducted 7 and 42 days after transplantation, and values were presented in relation to values obtained from comparably aged naïve animals by using the formula: % of naive = measured value after SCT/value measured in naive × 100.

Semiquantitative Histopathology of Lung, Gastrointestinal Tract, and Liver

On days +7 and +42 after transplantation, animals were sacrificed for histopathologic analysis. Lungs were removed, fixed in 4% paraformaldehyde dissolved in PBS, transferred into 70% ethanol after 48 hours, paraffin-embedded, and then 4-μm sections were obtained. Hematoxylin-eosin-stained lung sections from individual mice were coded without reference to mouse type and prior treatment and independently examined to establish an index of injury. Analysis was performed by light microscopy (Axioskop 2 plus, Carl Zeiss GmbH, Jena, Germany). Photomicrographs were acquired with the AxioCam HRc (Carl Zeiss GmbH) and processed with AxioVision Release 4.6.3 (Carl Zeiss GmbH). Lungs were evaluated for the presence of periluminal infiltrates or parenchymal pneumonitis using a semiquantitative scoring system as described previously [15,19].

Detailed gastrointestinal (GI) tract and liver histopathologic analyses were performed in a blinded fashion on days +7 and +42 after transplantation, as previously described [16,27].

Western Immunoblotting for Surfactant Protein A (SP-A)

Lung samples were homogenized in lysis buffer plus protease inhibitors (Tris-buffered saline [2×])+ EDTA pH 8, leupeptin [0.5 μg/mL], vanadate [0.5 mM], natriumfluoride [0.2 mM], aproptinin [2 μg/mL], beta-glycerolphosphate [10 mM], Na-pyrophosphate [0.2 mM], phenylmethylsulfonylfluoride [1 mM], nonidet [1%]) and centrifuged at 13,000 U/min for 15 minutes. Twenty-five micorgrams of protein extracts were separated on 15% SDS-polyacrylamide gels and transferred onto a nitrocellulose membrane (Schleicher & Schell, Dassel, Germany). Nonspecific binding was blocked using Tris-buffered saline/0.1% Tween 20 (TTBS) with 5% bovine serum albumin for 1 hour at room temperature. Subsequently, the blot was incubated with antibodies against surfactant protein A (1:2000; Chemicon, Chandlers Ford, UK) and actin (1:2000; Sigma-Aldrich, Taufkirchen, Germany) for 1 hour. Blots were washed in TTBS and incubated with peroxidase-conjugated goat-anti-rabbit antibody (1:2000, Dako, Glostrup, Denmark) for 30 minutes. Bands were visualized using chemiluminescence (ECL Western Blotting Analysis System and ECL Hyperfilm, Amersham Biosciences, Piscataway, NJ).

For quantification, blots were scanned (HP Deskjet F4180, Hewlett-Packard, Palo Alto, CA) and analyzed using the public domain NIH image program.

Statistical Considerations

All values are expressed as mean ± SEM. Statistical comparisons between groups were completed using the parametric independent sample *t* test if $n \geq 5$ or using the Mann-Whitney test if $n < 5$. For survival and cumulative incidence of pulmonary function changes, actuarial curves were obtained by Kaplan-Meier method and statistically compared using the log rank test. The *P* values were 2 sided, and a significance level of $\alpha = .05$ was used.

RESULTS

Lung Injury, Survival, and Clinical GVHD

Lethally irradiated B6D2F1 mice received SCT with 5×10^6 bone marrow and 6×10^6 splenocytes from either syngeneic (B6D2F1) or allogeneic (C57Bl/6) donors as described in Materials and Methods. Lung histopathology was obtained on day +7 and day +42 after transplantation, and pulmonary injury was determined by using a well-established scoring system [15,19]. Allogeneic recipients showed progressive lung injury from day +7, consistent with the development of interstitial pneumonitis, perivascular lymphocytic inflammation, and lymphocytic bronchiolitis until week 6, whereas lungs of mice receiving syngeneic SCT maintained normal histology (Figure 1a-b).

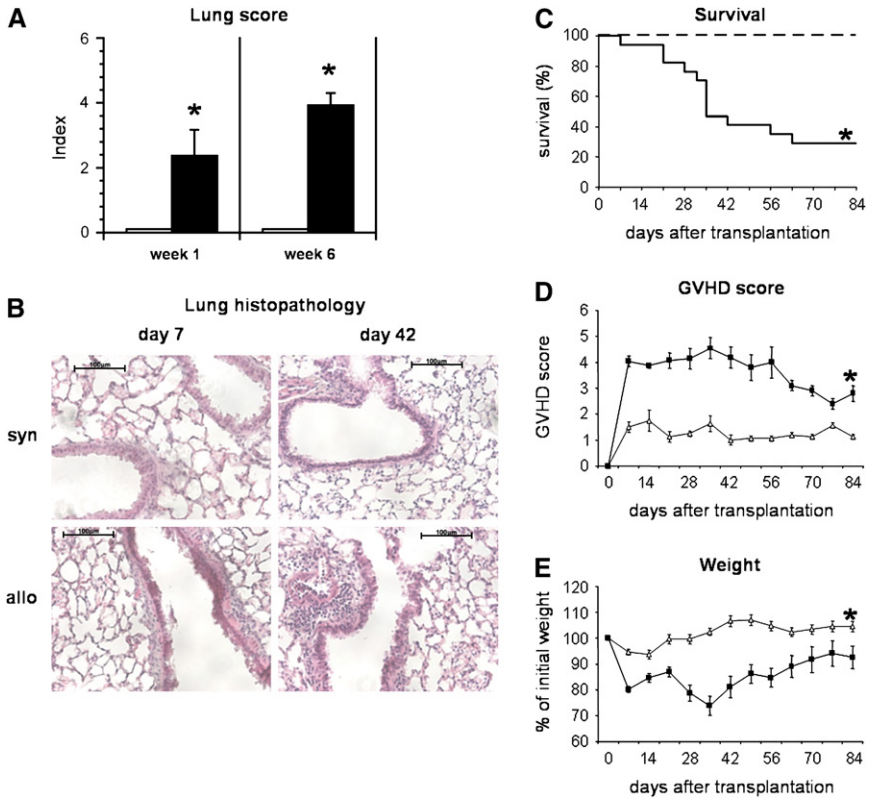

Figure 1. Lung injury, survival, and clinical GVHD. Lethally irradiated B6D2F1 mice received SCT with 5×10^6 bone marrow and 6×10^6 splenocytes from either syngeneic (B6D2F1) or allogeneic (C57Bl/6) donors as described in Materials and Methods. (A-B) Pulmonary injury was determined on days +7 and +42 after SCT by using a well-established scoring system and by histopathology; $^*P < .05$ (syngeneic [white bar] versus allogeneic [black bar]). Animals were monitored daily for (C) survival and weekly for (D) GVHD clinical scores and (E) weight. $^*P < .05$ (syngeneic [dotted line, open triangles] versus allogeneic [solid black line, filled squares]). Data are presented as mean ± SEM. Survival data are combined from 2 independent and comparable experiments; n (syngeneic) = 8, n (allogeneic) = 17 for survival. Histopatholoy data at week 1 are combined from 2 independent experiments (n [syngeneic] = 7, n [allogeneic] = 8) and at week 6 are combined from 3 independent experiments (n [syngeneic] = 7, n [allogeneic] = 16). $^*P < .05$.

These findings were confirmative of our previous studies using the same model of p-GVHD, in which we characterized the histopathologic changes and alterations in bronchoalveolar lavage cellularity and cytokine or chemokine expression [13,17-19].

Survival and the severity of clinical GVHD were monitored over 84 days after transplantation. Because of radiation conditioning toxicity, syngeneic recipients demonstrated minor changes in weight loss, fur texture, and mobility by week 1, but then rapidly regained body weight and completely recovered clinically. All of these animals survived (Figure 1c-e). In contrast, allogeneic recipients developed severe weight loss and clinical GVHD symptoms after SCT, and two-thirds of the animals succumbed by 12 weeks after allo-SCT, with the highest mortality rates between week 3 and week 6 (Figure 1c-e).

Changes in Minute Ventilation

The use of an unrestrained WBP to repeatedly assess basic breathing parameters in the same animal allowed us to test changes in MV, TV, RR, PIF, and PEF in surviving animals over the entire observation period of 12 weeks after transplantation.

MV is defined as the volume of gas that moves in and out of the lungs in 1 minute and is calculated by multiplying the exhaled TV by the RR. In syngeneic recipients MV dropped within the first 2 weeks after SCT to an average of 84% of pre-SCT values on day +14 and then went back to normal range values by week 5 (Figure 2a). TV was not decreased early after transplantation. Moreover, it increased over time in parallel to the weight gain observed in these animals (Figure 2b), and at the same time RR were decreased to maintain stable MV (Figure 2c). In contrast, allogeneic recipients demonstrated a dramatic decrease in MV by day +7 down to 57% of pre-SCT values, that persisted over time, and at week 12 MV only partially recovered to 73% of pre-SCT values in surviving animals (Figure 2a). In addition, TV and RR dropped significantly when compared to recipients of syngeneic SCT (Figure 2b-c). We then chose to assess cumulative incidences of severe reduction of MV, defined as MV ≤50% of pre-SCT values and of moderate to severe reduction of MV, defined as MV ≤60% of

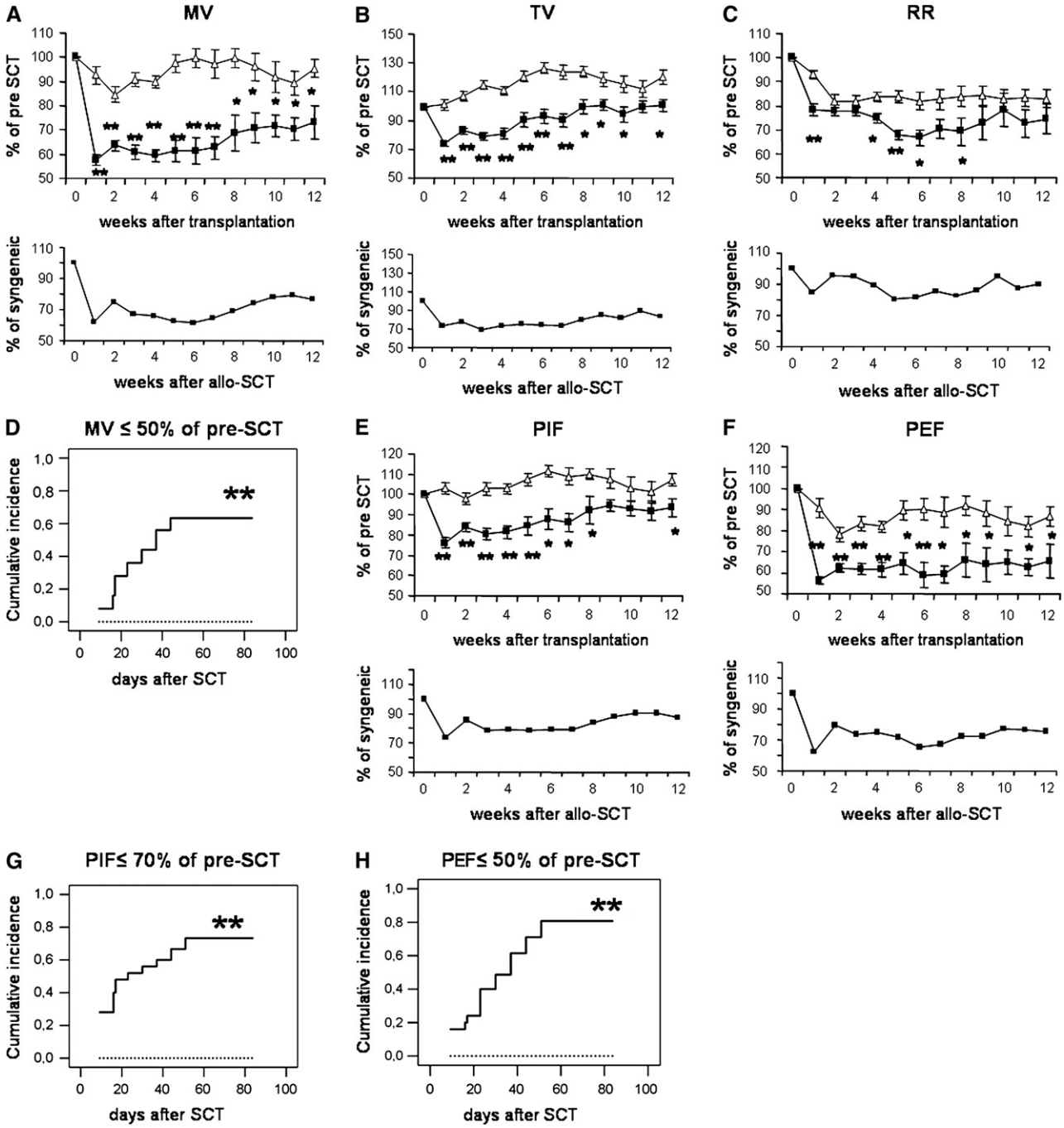

Figure 2. Changes in breathing patterns. Lethally irradiated B6D2F1 mice received SCT with 5×10^6 bone marrow and 6×10^6 splenocytes from either syngeneic (B6D2F1, dotted line, open triangles) or allogeneic (C57Bl/6, solid black line, filled squares) donors as described in Materials and Methods. Respiratory patterns were measured weekly by whole-body plethysmography in conscious, freely moving animals. To compare changes in breathing patterns of allogeneic recipients with syngeneic controls the following parameters were assessed: (A) minute ventilation (MV), (B) tidal volume (TV), (C) respiratory rate (RR), (D) cumulative incidences of MV ≤50% of pre-SCT, (E) peak inspiratory flow (PIF), (F) peak expiratory flow (PEF), (G) cumulative incidences of PIF ≤70% of pre-SCT, (H) cumulative incidences of PEF ≤50% of pre-SCT. For both syngeneic and allogeneic recipients, data shown are expressed as % of baseline values. In addition, changes in allogeneic recipients are demonstrated as % of syngeneic (A-C, E-F). Data are presented as mean ± SEM and are combined from 3 independent comparable experiments; initial n = 13 to 25 per group, minimal n at each time point ≥5; *$P < .05$; **$P < .001$.

pre-SCT values. These cut-off levels were chosen according to the observed MV changes (Figure 2a), and severe reduction of MV was defined as the next 10% interval below the lowest average, whereas moderate reduction was defined as the next 10% interval above the lowest average of MV. Syngeneic recipients never developed a moderate or severe reduction in MV. In contrast, by day +44 63.3% of allogeneic recipients

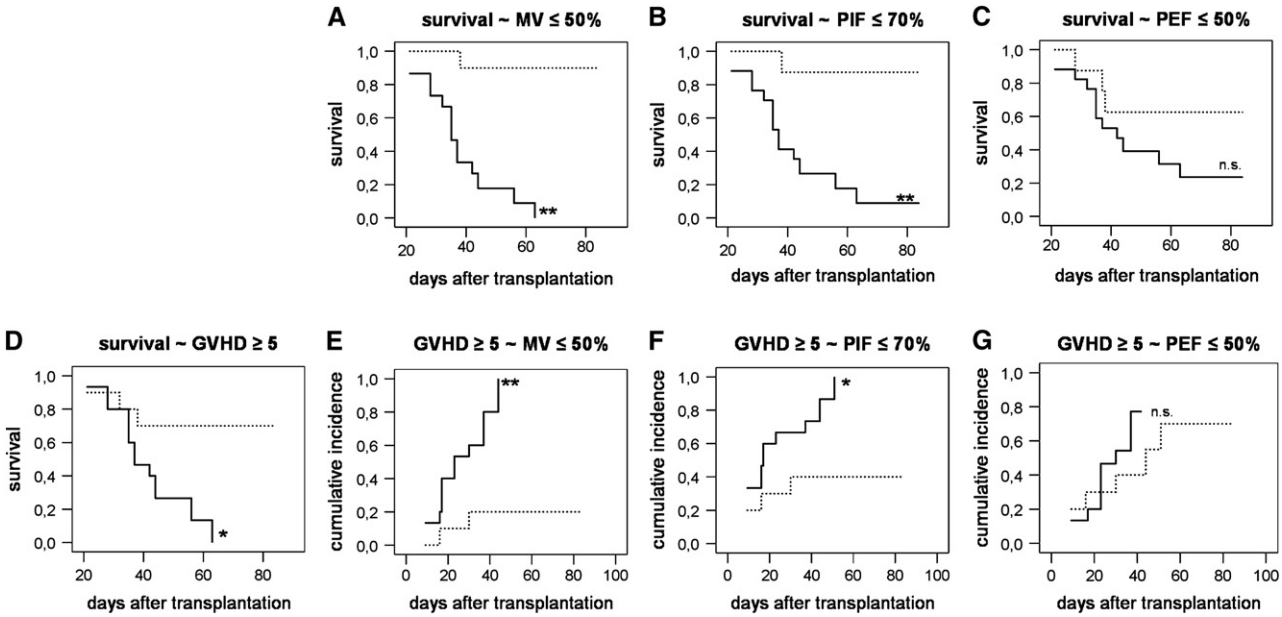

Figure 3. Changes in minute ventilation (MV) and peak inspiratory flow (PIF), but not peak expiratory flow (PEF), are associated with increased mortality after allogeneic SCT. (A-C) Increased mortality in allogeneic recipients given 5×10^6 bone marrow and 6×10^6 splenocytes was associated (A) with MV ≤50% (black solid line) versus MV >50% (black dotted line), (B) PIF ≤70% (black solid line) versus PIF >70% (black dotted line), but (C) not with reductions in PEF (PEF ≤50% [black solid line] versus PEF >50% [black dotted line]). (D) Overall survival in allogeneic recipients was directly related to clinical GVHD severity (GVHD ≥5: black solid line; GVHD <5: black dotted line). (E-G) Clinical GVHD scores ≥5 were associated with (E) MV ≤50% (black solid line) versus MV >50% (black dotted line), (F) PIF ≤70% (black solid line) versus PIF >70% (black dotted line), but (G) not with reductions in PEF (PEF ≤50% [black solid line] versus PEF >50% [black dotted line]). Data presented are combined from 3 independent comparable experiments; total n = 25; $^*P < .05$; $^{**}P < .001$.

developed a severe reduction in MV (P = .001; Figure 2d) and 90.7% had a moderate reduction in MV (P = <.001; data not shown).

Changes in PIF and PEF

Next, we analyzed whether changes in inspiration or expiration flow rates occurred along with the observed alterations in RR and TV. PIF was not affected after syngeneic SCT, and was significantly diminuished in allogeneic recipients when compared to pre-SCT values or to syngeneic controls (Figure 2e). Even more evident than the changes of PIF in allogeneic recipients, PEF dropped to 56% of pre-SCT values within the first week after allo-SCT and stayed reduced at levels <70% of pre-SCT values throughout the follow-up period (Figure 2f). In syngeneic recipients, PEF values were reduced by 22% within the first 2 weeks after SCT and then remained stable (Figure 2f). PEF in allogeneic recipients was significantly reduced at all times when compared with animals after syngeneic SCT. We then analyzed the cumulative incidences of severe reduction of PIF, defined as PIF ≤70% of pre-SCT values and of severe reduction of PEF, defined as PEF ≤50% of pre-SCT values. These cutoff levels were chosen according to the observed PIF and PEF changes (Figure 2e-f), and severe reduction of PIF or PEF was defined as the next 10% interval below the lowest average of PIF and PEF, respectively. Although syngeneic recipients did not develop either severe reductions of PIF or PEF (Figure 2g-h), severe reductions of PIF (P < .001) and PEF (P < .001) were seen in 73.3% and 80.7%, respectively, of allogeneic recipients by day + 51.

Increased Mortality in Animals Is Associated with Reductions in MV or PIF but not PEF

As allogeneic recipients developed significant changes in MV, PIF and PEF, we next tested, whether impaired MV, PIF, or PEF correlated with the increased mortality being observed between weeks 3 and 6 after allo-SCT with 5×10^6 bone marrow and 6×10^6 splenocytes. The strongest association with decreased survival was seen for MV; 88.2% of animals, in which MV deteriorated to ≤50% of pre-SCT values, died until day +44, and all of these animals died by day +100. In contrast, of those animals, in which MV remained better than 50% of pre-SCT values, 90% lived until the end of the observation period (P < .001) (Figure 3a). The development of severe reduction in PIF also correlated with increased mortality after allo-SCT (day 84: 91.2% versus 12.5%; P = .002) (Figure 3b), whereas changes in PEF did not (day 84: 76.5% versus 37.5%; P = 0.227) (Figure 3c).

One of the most important factors affecting survival after allo-SCT is the development of aGVHD. Therefore, we then analyzed if aGVHD severity

correlated with mortality and changes in breathing patterns. All animals developing maximum aGVHD ≥5 died by day +63, and 70% of animals with a maximum aGVHD score <5 survived until day +84 (P = .017; Figure 3d). All recipients of allo-SCT with aGVHD scores ≥5 developed severe reductions of MV until day +44 and of PIF until day +51, respectively, whereas until the end of the observation period MV and PIF values were severely decreased in only 20% and 30% of animals with aGVHD scores <5 (Figure 3e-f). In contrast, severe impairment of PEF was not associated with aGVHD severity, as severe reductions of PEF were found in 70.0% and 77.7% of mice, which had maximum aGVHD scores <5 and ≥5, respectively (Figure 3g).

We next wanted to know whether the observed changes in breathing patterns were because of intrinsic lung pathology or whether they rather reflected the clinically impaired general state of health after allo-SCT as indicated by severe clinical GVHD scores and weight loss. To address this question, we performed T cell titration experiments, in which animals received increasing numbers of donor spleen cells (1×10^6, 3×10^6, 6×10^6) to induce different degrees of GVHD severity, whereas numbers of bone marrow-derived stem cells were kept unchanged. As depicted in Table 1, elevated clinical GVHD scores directly related to the transplanted T cell dose both at week 1 (2.8 ± 0.2 versus 3.5 ± 0.0 versus 4.0 ± 0.2) and week 6 (2.7 ± 0.2 versus 3.1 ± 0.2 versus 4.2 ± 0.4) and, at week 1, lower clinical GVHD scores were paralleled by respective improvement in MV, PIF, and PEF. All of the animals receiving 1 or 3 million splenocytes developed mild to moderate clinical symptoms of GVHD, and none succumbed until day +42. In contrast, allo-SCT with 6 million splenocytes resulted in severe clinical disease and high mortality by this time point (Figure 1). Also, 6 weeks after SCT, MV, PIF, and PEF were decreased only in the latter group, whereas they were not altered in allogeneic recipients of 1 or 3 million splenocytes (Table 1), suggesting a direct relation between clinical GVHD related illness and changes in breathing patterns after allo-SCT.

Early clinical GVHD scores and mortality are predominantly driven by systemic inflammation because of the overexpression of inflammatory cytokines such as TNF or IFNγ rather than by donor T cell infiltration into GVHD target organs, which plays a major role during the cellular effector phase of GVHD later on [28-30]. Therefore, we next analyzed histopathologic changes in the GI tract, liver, and lung of syngeneic and allogeneic recipients 1 and 6 weeks after SCT. GVHD was induced by the infusion of either 1 or 6 million splenocytes at the time of transplantation. Consistent with prior findings [31], conditioning toxicity, early alloreactive T cell expansion, and inflammatory cytokine production induced significant GI tract injury early after allo-SCT, that declined by week 6 (Table 2). In contrast, no significant injury to the liver was observed by week 1, but was clearly evident by week 6 (Table 2). The lung demonstrated moderate changes by week +1, and injury progressed over time by week +6 (Table 2). Interestingly, the severity of histopathologic damage did not differ depending on the amount of allogeneic T cells being administered. Therefore, it seems highly likely that the susceptibility to individual mechanisms of the multipronged attack of post-SCT organ injury, including conditioning toxicity, alloreactive T cell responses, local cytokine production, NK cells, and others, differs

Table 1. *In Vivo Breathing Patterns Reflect Underlying GVHD-Induced Impairment of Physical Performance and Clinical Health*

		Week 1			Week 6		
Splenocyte Dose		**1×10^6**	**3×10^6**	**6×10^6**	**1×10^6**	**3×10^6**	**6×10^6**
GVHD score	***syn***	**1.2 ± 0.2**	**1.4 ± 0.4**	**1.5 ± 0.2**	**0.5 ± 0.0**	**0.5 ± 0.0**	**1.0 ± 0.2**
	allo	**2.8 ± 0.2†**	**3.5 ± 0.0†**	**4.0 ± 0.2†**	**2.7 ± 0.2†**	**3.1 ± 0.2†**	**4.2 ± 0.4†**
RR	***syn***	**99.3 ± 1.8**	**102.2 ± 4.8**	**93.0 ± 1.8**	**87.2 ± 4.5**	**93.6 ± 5.1**	**81.9 ± 3.6**
	allo	**94.2 ± 1.8**	**77.8 ± 4.0†**	**78.5 ± 2.5†**	**89.1 ± 2.5**	**91.5 ± 3.7**	**66.7 ± 3.4†**
TV	***syn***	**103.7 ± 2.5**	**107.7 ± 5.3**	**101.1 ± 3.7**	**117.6 ± 5.4**	**106.1 ± 6.2**	**126.4 ± 3.4**
	allo	**91.2 ± 1.9†**	**92.3 ± 2.1†**	**73.9 ± 1.7†**	**113.3 ± 6.0**	**112.7 ± 5.0**	**93.3 ± 4.9†**
MV	***syn***	**102.1 ± 3.6**	**109.6 ± 8.9**	**92.7 ± 3.7**	**98.3 ± 2.7**	**96.4 ± 3.4**	**99.6 ± 3.8**
	allo	**84.3 ± 1.3†**	**70.9 ± 4.1†**	**57.5 ± 2.3†**	**99.4 ± 5.4**	**100.4 ± 3 .1**	**61.5 ± 5.5†**
PIF	***syn***	**110.1 ± 2.4**	**110.8 ± 5.3**	**102.8 ± 2.8**	**111.6 ± 2.3**	**106.2 ± 3.0**	**111.5 ± 3.0**
	allo	**100.0 ± 1.1†**	**91.2 ± 2.5†**	**76.1 ± 2.5†**	**116.2 ± 5.1**	**116.0 ± 3.2**	**87.9 ± 5.2†**
PEF	***syn***	**97.8 ± 4.3**	**108.3 ± 10.7**	**90.6 ± 4.5**	**91.9 ± 3.4**	**92.5 ± 4.0**	**90.2 ± 5.0**
	allo	**77.2 ± 1.8†**	**69.3 ± 2.3†**	**56.3 ± 1.8†**	**96.3 ± 7.1**	**100.0 ± 4.0**	**58.8 ± 5.9†**

Lethally irradiated B6D2F1 mice were transplanted from syngeneic B6D2F1 or allogeneic C57BL/6 donors receiving 5×10^6 bone marrow cells and 1×10^6, 3×10^6, or 6×10^6 splenocytes. Clinical GVHD severity scores and respiratory patterns were measured on day +7 and day +42 as described in Materials and Methods. The following parameters were assessed: minute ventilation (MV), tidal volume (TV), respiratory rate (RR), peak inspiratory flow (PIF), and peak expiratory flow (PEF). For both syngeneic and allogeneic recipients, data shown are expressed as % pre-SCT values. Data are presented as mean ± SEM; n (syngeneic) = 5-13 per group; n (allogeneic) 8-25 per group.

†P < .01 comparing syngeneic versus allogeneic recipients at individual time points.

Table 2. *GVHD Target Organ Injury to the GI Tract Precedes Hepatic and Pulmonary Graft- versus-Host Disease*

		Week 1		Week 6	
Splenocyte Dose		1×10^6	6×10^6	1×10^6	6×10^6
Lung	syngeneic	0 ± 0	0 ± 0	0.6 ± 0.4	0 ± 0
	allogeneic	3.3 ± 0.7	2.4 ± 0.8	5.1 ± 0.4*	3.9 ± 0.4†
GI tract	syngeneic	0.1 ± 0.1	0.2 ± 0.2	0.6 ± 0.4	1.1 ± 0.3
	allogeneic	6.9 ± 0.8	8.8 ± 0.6	4.4 ± 0.5*	5.2 ± 0.5*
Liver	syngeneic	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	allogeneic	0 ± 0	0 ± 0	2.0 ± 0.4*	3.0 ± 0.5*

GI indicates gastrointestinal; GVHD, graft-versus-host disease.

Lethally irradiated B6D2F1 mice were transplanted from syngeneic B6D2F1 or allogeneic C57BL/6 donors receiving 5×10^6 bone marrow cells and 1×10^6 or 6×10^6 splenocytes. Histopathologic changes of the GI tract, liver, and lung were assessed 1 or 6 weeks after SCT. n (syngeneic): 4-11 per group; n (allogeneic): 6-16 per group.

*$P < .05$.

†$P = .05$ comparing scores at week 1 with scores at week 6 of identically treated animals.

between organs. Further, the clinical GVHD score and changes in breathing patterns rather reflect the sum of various independent factors than they can be attributed to specific GHVD related target organ injury, the latter specifically to the lung.

Changes in Vital Capacity, FEV, FEF, and Compliance

We next characterized whether the histopathologic changes observed in the lung after allo-SCT resulted in restrictive or obstructive alterations of pulmonary function because of intrinsic lung pathology. Restrictive lung injury was assessed by changes in VC, FVC, and compliance (Cchord) on day +7 and day +42 after SCT with 5 x 10^6 bone marrow and 6 x 10^6 splenocytes. At the same time points, changes in FEF and FEV were determined to test for air outflow obstruction and animals. Measurements were performed using an anesthesized mouse PFT plethysmograph as described in Materials and Methods, that, by applying passive ventilation to the sedated, nonself-breathing mouse, allows the assessment of pulmonary function changes, specifically lung volumes and flow measurements, which are because of direct lung pathology and independent of potentially confounding factors such as posture and muscle strength. Pulmonary function values were expressed in relation to naïve animals, and were compared between syngeneic and allogeneic groups. FVC, VC, and Cchord in animals receiving syngeneic SCT did not differ from untreated controls both 1 and 6 weeks after transplantation (Figure 4a-c). In contrast, allogeneic recipients showed significant decreases

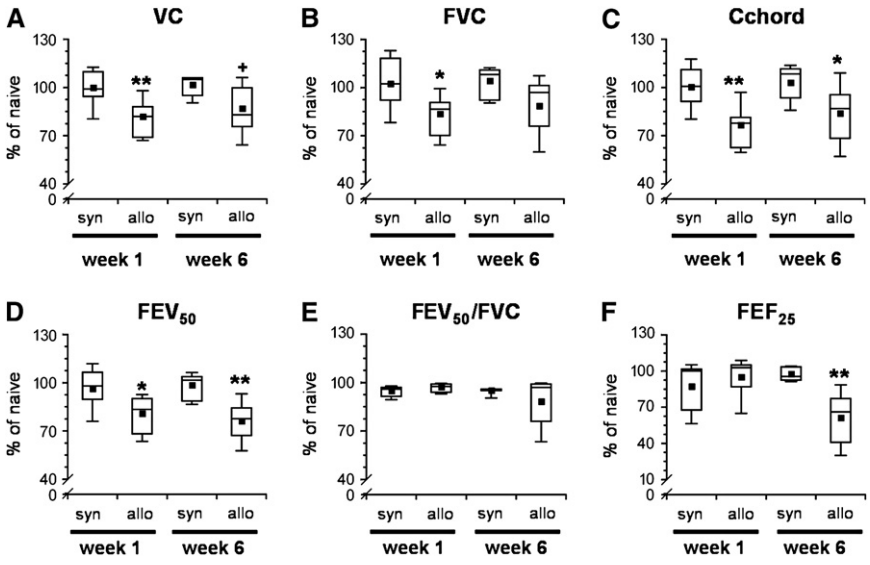

Figure 4. Changes in compliance, lung volumes, and flow rates. (A-F) Lethally irradiated B6D2F1 mice received SCT with 5×10^6 bone marrow and 6×10^6 splenocytes from either syngeneic B6D2F1 or allogeneic C57Bl/6 donors, and on day +7 and day +42 PFT were performed as described in Materials and Methods. Lung injury was assessed by changes in (A) VC, (B) FVC, (C) Cchord, (D) FEV_{50}, (E) FEV_{50}/FVC, (F) FEF_{25}. The lowest, second lowest, middle, second highest, and highest box points represent the 10th percentile, 25th percentile, median, 75th percentile, and 90th percentile, respectively. Means are represented by symbols. Data are presented as mean ± SEM and are combined from 2 independent comparable experiments; week 1: n = 7 to 10 per group, week 6: n = 7 to 9 per group. *$P < .05$, **$P < .01$, $^+P = .05$.

Table 3. *Changes in Lung Volumes and Flow Rates in Relation to Escalating Numbers of Allogeneic Donor T Cells*

		Week 1			Week 6		
Splenocyte Dose		1×10^6	3×10^6	6×10^6	1×10^6	3×10^6	6×10^6
VC	*syn*	107 ± 8.2	104 ± 4.6	99 ± 4.6	106 ± 5.7	106 ± 5.1	101 ± 2.8
	allo	89 ± 4.9	90 ± 4.0‡	81 ± 3.7†	101 ± 8.1	97 ± 6.0	86 ± 5.4‡
FVC	*syn*	111 ± 11.6	104 ± 4.5	102 ± 6.6	108 ± 3.2	110 ± 5.1	104 ± 4.0
	allo	92 ± 4.2	93 ± 4.4	83 ± 4.2*	101 ± 8.3	94 ± 9.4	88 ± 6.1
Cchord	*syn*	107 ± 9.8	99 ± 9.4	100 ± 4.6	108 ± 6.6	113 ± 5.8	103 ± 4.3
	allo	88 ± 5.5	88 ± 3.6	76 ± 4.5†	105 ± 9.8	102 ± 7.8	83 ± 6.4*
FEV_{50}	*syn*	104 ± 9.5	99 ± 3.1	96 ± 5.1	101 ± 2.3	106 ± 4.0	98 ± 3.5
	allo	86 ± 3.5	85 ± 7.4	80 ± 3.7*	88 ± 6.6	96 ± 4.2	76 ± 4.4†
FEV_{50}/FVC	*syn*	94 ± 1.5	96 ± 1.4	94 ± 1.3	94 ± 3.0	96 ± 1.1	95 ± 1.0
	allo	94 ± 2.5	98 ± 0.6	97 ± 0.8	89 ± 7.3	90 ± 4.2	88 ± 5.1
FEF_{25}	*syn*	109 ± 9.7	114 ± 6.5	87 ± 7.8	99 ± 15.6	84 ± 8.5	97 ± 2.3
	allo	96 ± 9.2	107 ± 6.4	94 ± 5.4	75 ± 14.7	69 ± 11.6	62 ± 7.1†

FVC indicates forced vital capacity; VC, vital capacity.

Lethally irradiated B6D2F1 mice were transplanted from syngeneic B6D2F1 or allogeneic C57BL/6 donors receiving 5×10^6 bone marrow cells and 1×10^6, 3×10^6, or 6×10^6 splenocytes. On day +7 and day +42 PFT were performed as described in Materials and Methods. Pulmonary function impairment was assessed by changes in VC, FVC, Cchord, FEV_{50}, FEV_{50}/FVC, and FEF_{25}. For both syngeneic and allogeneic recipients, data shown are expressed as % of naive. Data are presented as mean ± SEM; n (syngeneic) = 4-8 per group; n (allogeneic) 5-10 per group.

*$P < .05$

†$P < .01$

‡$P = .05$ comparing syngeneic versus allogeneic recipients at individual time points.

of FVC and VC and a significant reduction in Cchord as signs of restrictive lung function impairment as early as 7 days after SCT and by week 6, although at the latter time point decreases in only VC and Cchord reached statistical significance (Figure 4a-c).

We further analyzed, to which extent obstructive changes in air outflow developed in these animals. FEV_{50}, a parameter of mouse pulmonary function testing comparable to FEV_1 used for the assessment of pulmonary obstruction in human lung function testing, stayed within normal limits after syngeneic SCT when compared to untreated controls at 1 and 6 weeks after SCT (Figure 4d). In contrast, allo-SCT resulted in a significant drop of FEV_{50} both on day +7 and on day +42 (Figure 4d). In subjects with restrictive lung disease, FEV values can be lower than the predicted normal values because of a reduction of vital capacity volumes without the existence of real pulmonary obstruction. Therefore, we determined FEV_{50}/FVC, a parameter that allows the assessment of air outflow obstruction in the context of decreased FVC, and FEF_{25}, a parameter of obstructive changes in small airways. As depicted in Figure 4e, FEV_{50}/FVC was not reduced in allogeneic recipients 1 week after transplantation, but showed a decreasing trend at day +42 when compared to syngeneic controls and to untreated animals. However, this difference did not reach statistical significance (Figure 4e). Consistent with no changes in FEV_{50}/FVC 7 days after SCT, FEF_{25} did not differ between syngeneic and allogeneic recipients at this time point. However, FEF_{25} was significantly decreased by week +6, indicating a significant reduction in small airway flow rates by week +6 because of obstruction (Figure 4f). Taken together, these findings demonstrate that animals after allo-SCT developed severe restrictive but not obstructive changes by week 1 after transplantation, and that progressive inflammatory cell infiltrates in the lungs were paralleled by combined pulmonary restriction and the development of obstructive airway disease by week 6.

Using T cell titration experiments as described above, we next reduced but did not eliminate clinical aGVHD by infusing lower numbers of splenocytes (1×10^6 and 3×10^6). Lung volumes and flow rates again were assessed on day +7 and day +42, and none of the transplanted animals died before either time point of analysis (data not shown). Although statistically significant only in animals receiving 6×10^6 splenocytes, early analysis on day +7 revealed substantial decreases in VC, FVC, and Cchord consistent with pulmonary restriction in all 3 groups (Table 3). In contrast, at week 6, significant changes of Cchord ($P < .05$) and VC ($P = .05$) could be detected only in those animals receiving the highest dose of donor T cells. Decreases in FEF_{25} and FEV_{50} at day +42 also reached statistical significance only in those animals that were given 6 million splenocytes, but reductions in FEF_{25} indicative of obstructive small airway disease were clearly evident in all groups.

Reduced SP-A Levels in the Lung after allo-SCT

SP-A is produced by alveolar epithelial type II cells and, in addition to its antimicrobial function, it exerts suppressive effects on dendritic cell maturation and T cell proliferation [32-36]. Reduced SP-A levels

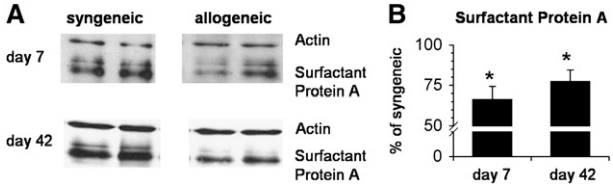

Figure 5. Reduced SP-A levels at 1 and 6 weeks after allo-SCT. (A-B) SP-A levels in the lungs of syngeneic and allogeneic recipients of 5 $\times$ 10^6 bone marrow and 6 $\times$ 10^6 splenocytes were determined at 1 and 6 weeks after transplantation as described in Materials and Methods. Data are combined from 3 comparable experiments (day 7: n = 11 animals per group; day 42: n = 9 syngeneic and 11 allogeneic recipients) and presented as mean $\pm$ SE. $^*P < .05$.

indicate an imbalance in surfactant regulation, and have been associated with early pulmonary injury after allo-SCT [37]. Therefore, we determined SP-A expression in the lung at 1 and 6 weeks following syngeneic or allogeneic transplantation. As depicted in Figure 5, SP-A expression in the lungs of allogeneic recipients was significantly reduced in comparison to syngeneic controls at both time points, suggesting, that decreased SP-A levels and disturbed surfactant balance contribute to restrictive pulmonary function changes and promote subsequent lung injury mediated by alloreactive T cells.

DISCUSSION

Beneficial effects of allo-SCT are counterbalanced by high mortality because of serious complications such as aGVHD and SCT-related injury to the lung. Historically, the lung has not been considered as a target organ of aGVHD, and lung injury was assumedly triggered by prior chemotherapy, conditioning regimen toxicity, and occult infections. However, experimental data as well as clinical experience today support the concept that the lung, in fact, is a critical organ in post-SCT immunology, as it is prone to injury by alloreactive donor immune responses [5,13-20,38-40], leading to p-GVHD. p-GVHD can present as interstitial und alveolar pneumonitis, perivascular lymphocytic inflammation, or lymphocytic peribronchiolitis [3-8,23], and histologic changes can be closely resembled in experimental models [14,15,17,20,21]. However, changes in pulmonary function have been only sporadically addressed in these models, and in vivo characterization of breathing patterns after allo-SCT has been completely missing.

The purpose of our study was to analyze changes in breathing parameters and pulmonary function after transplantation by using a well-established murine SCT model. In this system, development of pulmonary pathology is dependent upon the infusion of allogeneic T cells along with the pretransplant radiation dose and involves the recruitment of alloreactive $CD4^+$ and $CD8^+$ T cells to the lung [13,17,41]. Importantly, lung injury that develops in this model is defined by several features that characterize human disease including robust pulmonary infiltrates, increased capillary permeability, and inflammatory cytokine release [13,17].

Using this system, we chose 2 approaches to assess how pulmonary physiology is affected after allo-SCT. First, measurement of baseline breathing parameters after transplantation was performed by whole-body plethysmography. This method allowed us to evaluate the living, unstressed animal in the absence of noninvasive or invasive ventilation, arranging for a setting that simulates baseline respiratory function in the nonventilated out- or inpatient subject after clinical SCT. An extended follow-up until day +84 enabled us to better assess how survival is affected by the development of p-GVHD in a model, in which lung injury histologically has fully evolved by day +42 [17]. Our results demonstrated that severe clinical distress because of the development of aGVHD in allogeneic recipients was mirrored by severe impairment of MV and peak flows during inspiration and expiration. In clinical patients, beginning or manifest respiratory insufficiency or other factors such as pain, fever, or stress, leads to tachypnoeae in combination with decreased, normal, or increased TVs to ensure sufficient MV and oxygenation. However, in our study, allogeneic recipients displayed a significant decrease in both breathing frequency and TV, most likely because of a combination of aGVHD-related systemic weakness and changes in air flow patterns. Inspiration as an active procedure of diaphragm and respiratory muscles directly depends on the ability to expand the lung against elastic recoil, air flow resistance, and tissue resistance of lung and thorax [42]. Expiration in the resting state and healthy lung is exclusively a passive procedure mediated by the elastic recoil of lung and thorax, but in the context of respiratory distress is actively supported by respiratory muscles. Therefore, our data suggest that animals after allo-SCT in a severely reduced state of health are not capable to maintain or increase baseline breathing frequencies, TVs, and inspiratory or expiratory flow rates to achieve compensation for a higher need of oxygen. Instead, exertion of diaphragm and respiratory muscles in combination with hunched posture and functional restrictive and obstructive changes after allo-SCT cause severe ventilation impairment, which is directly associated with increased mortality (Figure 3). Severely hindered ventilation was directly associated with clinical aGHVD severity, and partial recovery of ventilation parameters in surviving animals following week +8 post-SCT along with decreased aGVHD scores reflects a population bias caused by mortality of severely sick animals and survival of less sick animals, respectively.

An initial drop in MV was also seen in animals after syngeneic SCT, which can be explained by the effects of conditioning toxicity on general strength and health

in these animals. In contrast to allogeneic recipients, however, reduced ventilation after syngeneic SCT was not associated with any mortality.

In a second approach, we evaluated compliance as well as static and dynamic lung volumes as indicators of restrictive or obstructive pulmonary function changes after SCT. Command-controlled test procedures cannot be performed in mice as they can in humans. Therefore, the underlying technique involves passive ventilation of the sedated, not spontaneously breathing animal in combination with a high-end plethysmograph, and, in contrast to our first approach, parameters obtained do not depend on active breathing maneuvers of the transplanted animal and directly reflect intrinsic lung pathology. Both static lung volumes including VC, FVC, and compliance were not affected in syngeneic recipients. Also, no pulmonary obstruction developed in these animals. In contrast, transplantation of allogeneic T cells led to significant pulmonary restriction as early as 1 week after SCT. These findings are comparable to results obtained by using a different mouse model of lung injury after allo-SCT, when a reduction of total lung capacity [14,24] and compliance [14,25,26] were observed around the same time along with interstitial thickening and mild to moderate perivascular inflammatory cell infiltrates [14]. Respective histopathologic changes early after allogeneic SCT were seen in our study in about one-third of animals, whereas restrictive pulmonary function changes were found in >80% of these animals, suggesting that impaired pulmonary function may reflect early histologic changes that cannot yet be identified by light microscopy [14] and precede obvious pulmonary inflammation. Further, at this point it is still unclear whether histology and lung function changes seen this early after allo-SCT in both models indeed present acute noninfectious interstitial pneumonitis after allo-SCT, historically defined as early IPS [4] as they could also mirror PERDS or the effects of conditioning toxicity in close interplay with alloreactive T cell expansion in secondary lymphoid organs and systemic inflammation [30,41]. TNF:TNF receptor type I (TNFRI) interactions have proved to be critical in the development of aGVHD and early mortality after allo-SCT [30,31,43]. Shukla and colleagues [25] recently demonstrated that interrupting TNF:TNFRI signaling in allo-SCT recipients resulted in improved early post-SCT survival that was associated with decreased pulmonary edema and improved lung compliance on day 7 after SCT. In the same animals, however, cellular infiltration into the lung as a classical characteristic of IPS was actually stronger than that seen in those allogeneic recipients, in which TNF:TNFRI signaling was intact along with increased pulmonary edema and higher mortality [25]. This suggests that the observed lung injury at 1 week after transplantation may rather resemble acute disturbances in endothelial and epithelial barrier function, similar to those seen in PERDS or triggered by systemic inflammation, and does not necessarily reflect cellular pneumonitis. Decreased SP-A levels early after allo-SCT may, however, promote the development of alloreactive T cell-mediated injury to the lung because of a lack of its suppressive effects on dendritic cell maturation and T cell proliferation [32-36].

In our study, restrictive pulmonary function changes at week +1 after allo-SCT occurred in recipients of low, medium, and high numbers of donor T cells, and although a statistical significance was only shown for those animals receiving the highest T cell dose, reductions in VC, FVC, and Cchord in all groups but not in syngeneic controls demonstrate that the development of early pulmonary restriction is at least partially T cell dependent and involves critically cocontributing lung-alloantigen-independent factors, for example, radiation-mediated endothelial and epithelial injury and systemic cytokine expression.

Parallel to the development of parenchymal pneumonitis and of periluminal lung infiltrates around airways and vessels, progressive air outflow obstruction in addition to restrictive pulmonary function changes occurred in surviving allogeneic recipients by week 6. The development of restriction 6 weeks after allo-SCT was directly related to the T cell dose, as significant restrictive changes of pulmonary function occurred only in animals receiving the highest number of splenocytes. Obstructive pulmonary function changes also seemed T cell dose dependent, as reductions in FEF_{25} and FEV_{50} only reached significance with the highest dose of T cells given, but were also detectable in those groups treated with low or medium numbers of donor splenocytes and in the absence of GVHD-related mortality. Panoskaltsis et al. [44] recently described a murine model, in which obstructive pulmonary changes occurred around 2 months after allo-SCT, accompanied by periluminal donor cell infiltrates and by histologically proved obliterative bronchiolitis in a minority of animals. Therefore, lymphocytic peribronchiolitis plus air outflow obstruction in our study likely present a form of p-GVHD, that in some cases gives rise to the development of bronchial luminal obliteration, whereas manifest alveolar and interstitial cell infiltrates along with inflammatory cytokine expression [12,13,16,17] resemble classical IPS as an acute form of p-GVHD, leading to increased mortality.

In summary, our data demonstrate a close correlation between the development of clinical GVHD, p-GVHD, and impaired ventilation after allo-SCT. Respiratory dysfunction, because of both alloimmune-mediated injury to the lung and because of aGVHD-related reductions in the general state of health, are directly associated with increased mortality, emphasizing the lung as a critical GVHD target organ

in SCT immunology. Further, in addition to histopathology, BALF cellularity and cytokine expression, pulmonary function testing in experimental models of p-GVHD provides a powerful tool to assess the severity of lung injury, as it directly provides important information on p-GVHD-related clinical and pathophysiologic ramifications, and may be additionally used to monitor the effectiveness of experimental therapeutic approaches.

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